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19-Nor-1α,25-dihydroxyvitamin D_2 (paricalcitol): effects on clonal proliferation, differentiation, and apoptosis in human leukemic cell lines

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Abstract *Purpose*: 19-Nor-1α,25-dihydroxyvitamin D_2 (paricalcitol) is an analogue of $1,25(OH)_2D_3$ with reduced calcemic effects that is approved for the suppression of parathyroid hormone in chronic renal failure. Paricalcitol has recently been reported to have anticancer activity in prostate cancer. In order to explore paricalcitol as a potential agent against leukemia, we tested its effects on HL-60 and U937 leukemia cell lines. *Methods*: We studied cellular differentiation via expression of CD11b and CD14 surface antigens using flow cytometry, and via the nitroblue tetrazolium (NBT) assay. Cell cycle was analyzed using propidium iodide staining. Apoptosis was assessed with the annexin V assay. Cellular proliferation was determined via colony inhibition on semisolid medium. *Results*: Paricalcitol induced the maturation of HL-60 and U937 cells, as shown by increased expression of CD11b differentiation surface antigen. CD14 showed increased expression in HL-60 but not in U937 cells. After exposure to paricalcitol at 10^{-8} *M* for 72 h, the ability of HL-60 cells to reduce NBT was markedly increased. Conversely, U937 cells were unchanged. Paricalcitol inhibited colony formation of both HL-60 and U937 cell lines in semisolid medium after a 10-day incubation (estimated IC_{50} of 3×10^{-8} *M* in HL-60 cells and 4×10^{-8} M in U937 cells). Paricalcitol at 10^{-8} *M* and 10^{-7} *M* caused a significant dose- and time-dependent increase of apoptosis in HL-60 cells ($P<0.05$). In both HL-60 and U937 cells, exposure to 10^{-7} *M* paricalcitol for 72 h increased the number of cells in G_0/G_1 phase, and decreased the number of cells in S phase. *Conclusions*: Paricalcitol inhibits colony formation, induces maturation and causes cell cycle arrest in HL-60 and U937 cells. Additionally, paricalcitol induces apoptosis in HL-60 cells. These findings support the further evaluation of paricalcitol as an antileukemia agent.

Keywords Paricalcitol · HL-60 · U937 · Apoptosis · Differentiation · Experimental therapeutics

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Introduction

The hormonal form of vitamin D, $1,25(OH)_2D_3$ (calcitriol), inhibits the proliferation and induces the differentiation of normal and leukemic myeloid cells into monocytes (Abe et al. 1981; McCarthy et al. 1983). $1,25(OH)_2D_3$ and its synthetic analogues appear to exert their growth-inhibitory effects via regulation of cell cycle progression (Jiang et al. 1994; Liu et al. 1996; Wang et al. 1996). Typically, treatment of cells with $1,25(OH)_2D_3$ causes an arrest of cells in G_1 phase, resulting in a decreased number of cells in S phase, with an increase of cells in G_0/G_1 (Kawa et al. 1996; Wang et al. 1996; Blutt et al. 1997; Segaert et al. 1997; Simboli-Campbell et al. 1997; Wu et al. 1997; Park et al. 2000; Seol et al. 2000). This change is associated with alterations in the expression of cell cycle regulators and other genes. In most cell types, growth inhibition and maturation accompany each other.

The antiproliferative and differentiating effects of calcitriol suggest a therapeutic role for the drug in hematological malignancies. Using calcitriol and its analogues, promising responses have been observed in some clinical studies (Arlet et al. 1984; Motomura et al. 1991;

Slapak et al. 1992; Mellibovsky et al. 1998), but not in all (Mehta et al. 1984; Koeffler et al. 1985; Richard et al. 1986). The principal toxicity of these drugs is hypercalcemia (Mehta et al. 1984; Koeffler et al. 1985; Richard et al. 1986). Consequently, numerous vitamin D analogues have been synthesized in order to identify compounds with less calcemic and/or greater antineoplastic activity than calcitriol (Zhou et al. 1989; Lee et al. 1996; Munker et al. 1996; Pakkala et al. 1997; Asou et al. 1998; Hisatake et al. 2001). Recently, a new class of vitamin D analogues, 19-nor-vitamin D_3 compounds, have been described as potent inhibitors of proliferation and inducers of differentiation (Asou et al. 1998).

One 19-nor analogue, 19-nor-1α,25-dihydroxyvitamin D_2 (paricalcitol, Zemplar, Abbott Laboratories, Chicago, Ill.) is approved in the US for the treatment of secondary hyperparathyroidism in chronic renal failure (Martin et al. 1998). In vitamin D-deficient rats, paricalcitol is 10 to 100 times less active than calcitriol in stimulating intestinal Ca^{++} transport or Ca^{++} mobilization from bone (Slatopolsky et al. 1997) and has less bone-resorbing activity than calcitriol in vitro (Holliday et al. 2000). Paricalcitol is as effective as calcitriol in its ability to transactivate the vitamin D receptor (VDR) and to inhibit the proliferation of prostate cancer lines and prostate cancer cells in primary culture (Chen et al. 2000). These findings have led to an ongoing phase I/II trial of paricalcitol in advanced prostate cancer (F.M. Torti, 2002, personal communication).

In the study reported here, we examined the effects of paricalcitol on cell maturation, inhibition of colony formation, cell cycle arrest, and apoptosis in two leukemic cell lines, the human monocytic cell line U937, and the myeloid leukemia cell line HL-60. In human leukemia cells, the onset of differentiation towards monocytes is accompanied by an increased expression of the surface antigens CD11b and CD14, and the phagocytotic activities of the cells, e.g. induction of superoxide production, are enhanced. We therefore measured the effects of paricalcitol on the expression of CD11b and CD14, and on superoxide production. We also tested the effect of paricalcitol on clonal proliferation of mononuclear cells derived from healthy bone marrow.

Methods

Cells and compounds

HL-60 and U937 cells were obtained from the American Type Culture Collection (Rockville, Md.) and were maintained in RPMI-1640 with 10% fetal calf serum and 100 U/ml penicillin, 100 μg/ml streptomycin in an incubator at 37°C in an atmosphere containing 5% CO_2. Paricalcitol was provided by Abbott Laboratories, and $1,25(OH)_2D_3$ (calcitriol) was purchased from Biomol Research Laboratories (Plymouth Meeting, Pa.). The drugs were protected from light and dissolved in absolute ethanol and were kept at −70°C. A bone marrow specimen was obtained from a healthy human volunteer after obtaining informed consent. Light density mononuclear cells were obtained with a one-step density centrifugation with Fico/Lite-LymphoH (density 1.077; Atlanta Biologicals, Atlanta, Ga.).

Flow cytometry analysis of maturation antigens

For analysis of cellular differentiation, expression of cell surface antigens was determined using immunofluorescence staining. Cells were incubated for 4 days with different concentrations of drug in a 24-well tissue culture plate (Costar, Corning, Corning, N.Y.) in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. After 48 h, fresh medium containing the same concentration of drug was added to each well. Cells were harvested, counted and the viability was assessed using trypan blue. Cells were incubated for 10 min with bovine serum albumin as a blocking agent and then stained using mouse anti-human CD11b antibody conjugated with *R*-phycoerythrin (RPE) or mouse anti-human CD14 antibody conjugated with RPE (DAKO, Carpinteria, Calif.). Control studies were performed with mouse RPE-conjugated IgG_1 or IgG_{2a} isotype antibodies (DAKO). Cells were analyzed using a FACSTAR Plus flow cytometer (Becton Dickinson).

Analysis of differentiation

Differentiated HL-60 cells, like normal monocytes, produce superoxide anions (O_2^-) when stimulated with 12-*O*-tetra-decanoylphorbol-acetate (TPA) (Newburger et al. 1979). After a 4-day incubation, 1×10^6 cells/condition were harvested by centrifugation at 250 *g* for 7 min. After resuspension in medium, half of the cell suspension was used to make cytospin slides for morphological assessment and comparison to the cells exposed to nitroblue tetrazolium (NBT). The other half was mixed 1:1 with a solution of Dulbecco's phosphate-buffered saline (DPBS) containing reagents to achieve final concentrations of 0.5 mg/ml NBT (Sigma, St. Louis, Mo.), 162 n*M* TPA (Sigma), 10% FBS and 0.5×10^6 cells/ml. After 25 min in humidified air containing 5% CO_2 at 37°C, the reaction was stopped by placing the tubes on ice. Cytospin slides were made for each condition and the slides were stained with Wright stain (Sigma, St. Louis, Mo.). A minimum of 100 cells were counted and the percentage of NBT-positive cells was assessed under light microscopy for each experimental point.

Apoptosis analysis

Human recombinant annexin V labeled with fluorescein isothiocyanate (FITC) was used to detect apoptotic cells. The assay was performed according to the manufacturer's recommendations (Alexis Corporation, San Diego, Calif.). Briefly, cells were washed in PBS, resuspended in a binding buffer (10 m*M* Hepes/NaOH, pH 7.4, 140 m*M* NaCl, 2.5 m*M* $CaCl_2$), incubated with annexin-FITC for 10 min in the dark, and propidium iodide (PI) was added prior to two-color flow cytometry analysis. The statistical significance of differences between groups was analyzed by a *t*-test for independent samples.

Cell cycle analysis

Cell cycle analysis was performed on HL-60 cells treated with either paricalcitol or $1,25(OH)_2D_3$ for 72 h. The cells (1×10^6) were harvested, washed twice with PBS, and stained with 50 μg/ml PI, 37 μg/ml RNase, and 0.6% NP40 in a 3.6-m*M* citrate buffer (Mayfield et al. 2001). Cell cycle analysis was performed from the list mode data of the FACSTAR Plus flow cytometer using a modeling program (MODFIT, Verity Software House, Topsham, Me.). The results were analyzed by multivariate analysis of variance.

Methylcellulose assay for colony formation

HL-60 and U937 cells in logarithmic growth phase were harvested and mononuclear cells were isolated from 2 ml bone marrow

collected from a healthy volunteer. The mononuclear cells from the bone marrow, and HL-60 and U937 cells were plated at 1 ml per 35-mm dish at concentrations of 50,000, 3,000 and 2,000 cells/ml, respectively. The medium contained 1% methylcellulose in Iscove's MDM, 30% FBS, 1% bovine serum albumin, 10^{-4} *M* 2-mercaptoethanol, 2 m*M* L-glutamine, 50 ng/ml rh-stem cell factor, 10 ng/ml rh-GM-CSF, 10 ng/ml rhIL-3, and 6 U/ml erythropoietin (Methocult GF H4534; Stem Cell Technologies, Vancouver, BC). Calcitriol and paricalcitol were added to the semisolid medium to the desired final concentrations. Ethanol was used in the control plates. The plates were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Cell colonies (≥40 cells) were counted under an inverted microscope after 10 days of incubation for the HL-60 and the U937 cell lines. The colonies formed from colony-forming unit granulocyte-macrophage (CFU-GM), colony-forming unit granulocyte/erythroid/macrophage and megakaryocyte (CFU-GEMM) and burst-forming unit erythroid (BFU-E) were counted after 14 days for the bone marrow mononuclear cells. Each experiment was performed in triplicate. In both cell lines colony inhibition by calcitriol and paricalcitol was compared using analysis of variance.

Results

Effect of paricalcitol on maturation of HL-60 and U937 cells

CD11b and CD14 antigens are markers of maturation in leukocytes. CD11b, one of the β_2-integrins, is the α subunit of a heterodimeric surface glycoprotein with a role in inflammatory and phagocytic responses. CD11b is expressed mainly on mature monocytes, on macrophages, on most polymorphonuclear leukocytes, and on a minor subset of B lymphocytes. CD14, the lipopolysaccharide receptor, is thought to play a similar

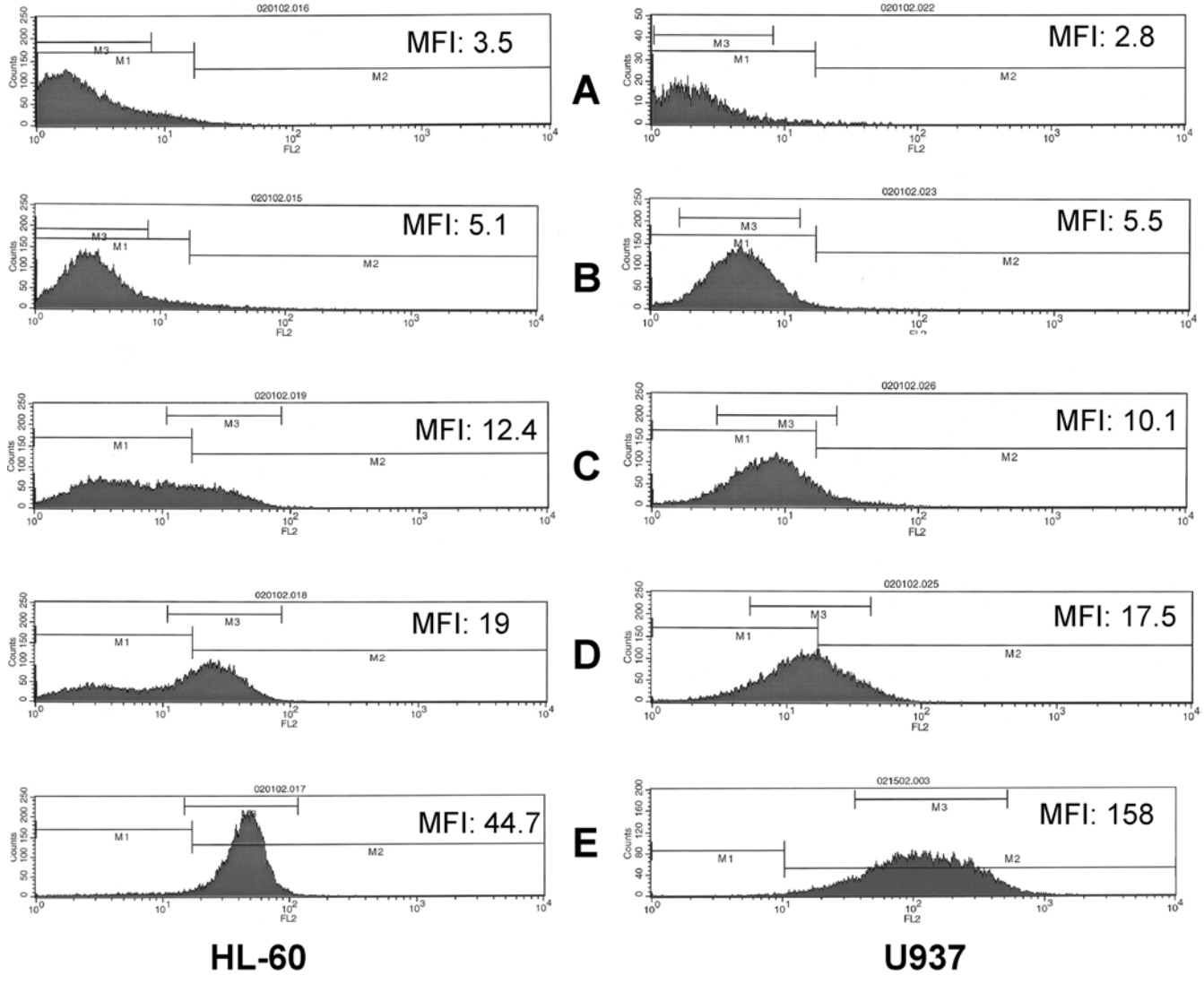

Fig. 1A–E Expression of CD11b antigen on HL-60 and U937 cells incubated with different concentration of paricalcitol (19-nor-1α,25-dihydroxyvitamin D_2) for 4 days. **A** Cells after incubation with no drug with nonspecific RPE-labeled mouse antibody. **B** Cells after incubation with no drug with RPE-labeled mouse anti-human CD11b antibody. **C** Cells after incubation with 10^{-9} *M* paricalcitol with PE-labeled mouse anti-human CD11b antibody. **D** Cells after incubation with 10^{-8} M paricalcitol with PE-labeled mouse anti-human CD11b antibody. **E** Cells after incubation with 10^{-7} *M* paricalcitol with RPE-labeled mouse anti-human CD11b antibody. The mean fluorescence intensity (MFI) of the cells is shown for each experimental condition

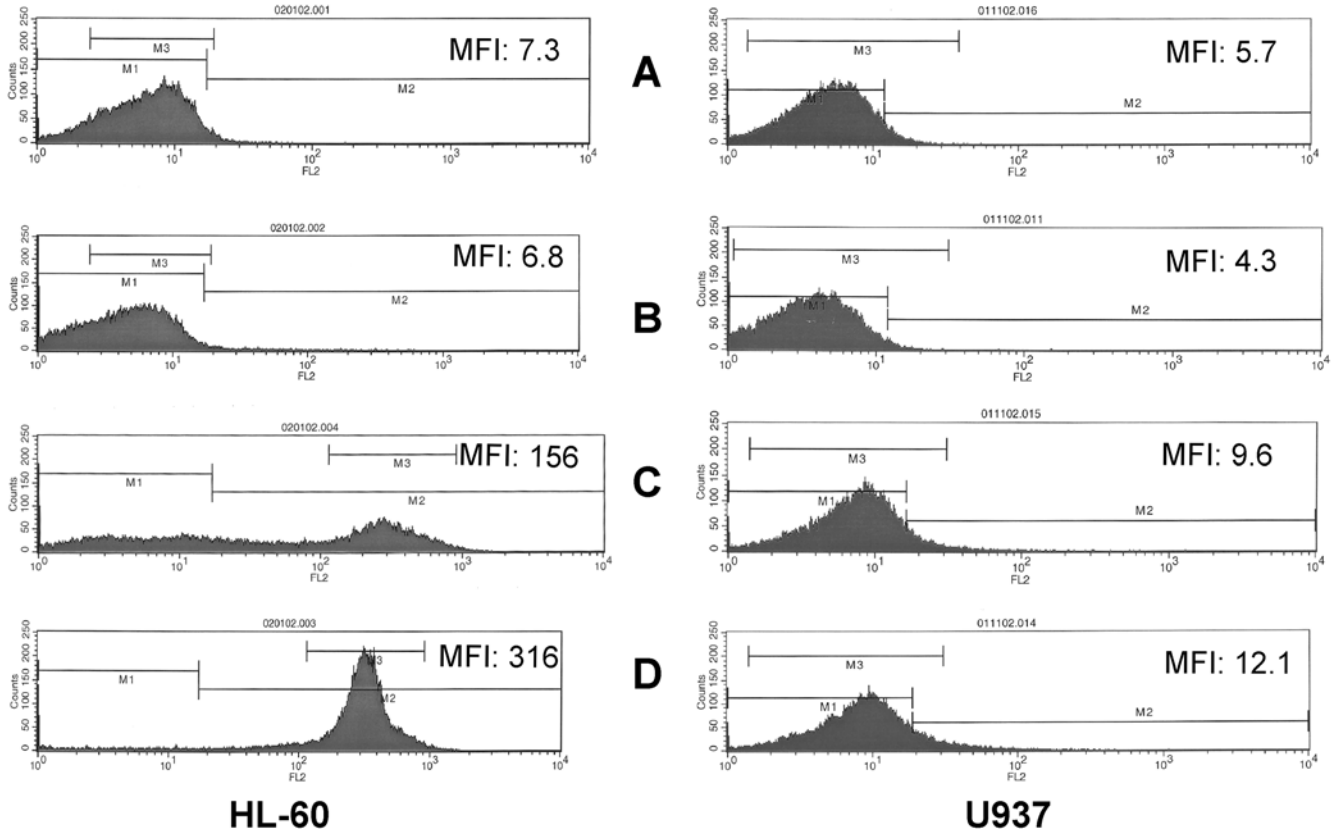

Fig. 2A–D Expression of CD14 antigen on HL-60 and U937 cells incubated with different concentration of paricalcitol (19-nor-1α,25-dihydroxyvitamin D_2) for 4 days. **A** Cells after incubation with no drug with nonspecific RPE-labeled mouse antibody. **B** Cells after incubation with no drug with RPE-labeled mouse anti-human CD14 antibody. **C** Cells after incubation with 10^{-8} *M* paricalcitol with RPE-labeled mouse anti-human CD14 antibody. **D** Cells after incubation with 10^{-7} *M* paricalcitol with RPE-labeled mouse anti-human CD14 antibody. Mean fluorescence intensity (MFI) of the cells is shown for each experimental condition

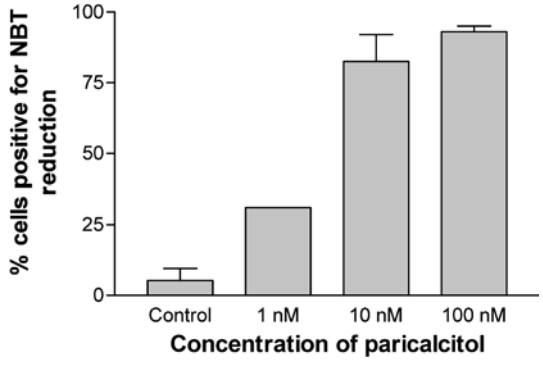

Fig. 3 Effect of paricalcitol on reduction of NBT by HL-60 cells. HL-60 cells were treated for 72 h with different concentrations of paricalcitol (10^{-9}, 10^{-8} or 10^{-7} *M*), then analyzed for reduction of NBT. The results are expressed as percentage of cells that reduced NBT

role to CD11b, and has a significant role in noninflammatory phagocytosis of apoptotic cells (Devitt et al. 1998). CD14 is strongly expressed on the surface of monocytes and weakly on the surface of most granulocytes and tissue macrophages. Exposure of the HL-60 and U937 cells to paricalcitol at 10^{-9}, 10^{-8} and 10^{-7} *M* for 4 days produced 25%, 50% and 93% cells expressing CD11b in HL-60, and 11%, 39% and 99% cells expressing CD11b in U937 cells (Fig. 1). CD14 expression differed between the two cell lines: U937 showed no expression of CD14 after a 4-day exposure to paricalcitol, while 29%, 62% and 95% of HL-60 cells expressed CD14 after exposure to paricalcitol at 10^{-9}, 10^{-8} and 10^{-7} *M* (Fig. 2).

The ability to reduce NBT and the number of cells that have undergone myelocyte maturation show a good correlation. After a 72-h treatment of HL-60 cells with paricalcitol at 10^{-9}, 10^{-8} and 10^{-7} *M*, the percentage of cells able to reduce NBT rose from 5.5% to 31%, 83% and 93% (Fig. 3). Conversely, U937 cells did not develop granules (data not shown).

Effect of calcitriol and paricalcitol on induction of apoptosis in HL-60 and U937 cells

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, exposing it to the external environment. Annexin V has high affinity for PS and can identify cells with exposed PS (Willingham 1999). PI is used as a viability probe. Cells staining positive for annexin V-FITC and negative for PI are undergoing apoptosis.

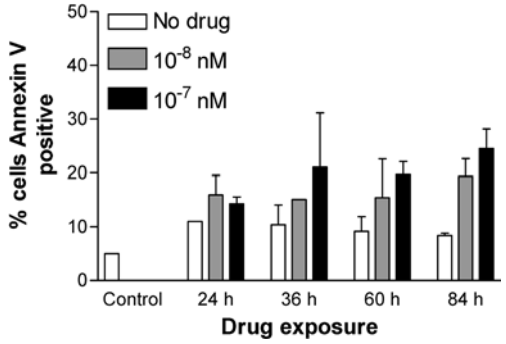

Fig. 4 Induction of apoptosis by paricalcitol in HL-60 cells. HL-60 cells were incubated with 10^{-8} *M* or 10^{-7} *M* paricalcitol for 24, 36, 48 and 84 h. Cells were then analyzed by flow cytometry to determine the percentage of cells which were annexin V-FITC-positive and PI-negative, indicating cells undergoing apoptosis

Table 1 Cell cycle distribution of U937 and HL-60 cells after a 72-h incubation with different concentrations of paricalcitol (10^{-8} and10^{-7} *M*). The percentage of cells in the different cell cycle phases is presented as determined by flow cytometry

	U937 cells			HL-60 cells		
	G_0/G_1	G_2/M	S	G_0/G_1	G_2/M	S
No drug	49	10	41	56	11	33
10^{-8} *M*	52	10	39	65	12	23
10^{-7} *M*	59	7	34	78	8	14

Paricalcitol (at both 10^{-8} *M* and 10^{-7} *M*) significantly increased the percentage of HL-60 cells undergoing apoptosis ($P < 0.05$, Fig. 4), but induced no significant change in U937 cells. Calcitriol did not cause a significant increase in the number of cells undergoing apoptosis in either cell line (data not shown).

Analysis of cell cycle

Calcitriol causes a decrease in the number of HL-60 and U937 cells in S phase and an increase in the number of cells in G_0/G_1 phase (Studzinski et al. 1985; Godyn et al. 1994; Wang et al. 1996; Rots et al. 1999). Similarly, paricalcitol induced a decrease in the number of cells in S phase and a simultaneous increase in the number of cells in G_0/G_1 phase ($P = 0.03$ in U937 cells and $P = 0.001$ in HL60 cells). The changes in cell cycle distribution of HL-60 and U937 cells after a 72-h exposure to 10^{-8} *M* or 10^{-9} *M* calcitriol and paricalcitol are shown in Table 1.

Inhibition of colony formation

Both calcitriol and paricalcitol inhibited colony formation of HL-60 and U937 cells in semisolid medium after a 10-day incubation (Fig. 5). This inhibition was concentration-dependent. For paricalcitol, the estimated 50% inhibitory concentrations (IC_{50}) were 3×10^{-8} *M* for HL-60 and 4×10^{-8} *M* for U937 cells. For calcitriol, equivalent values were 5×10^{-8} *M* for HL-60 and 8×10^{-8} *M* for U937 cells.

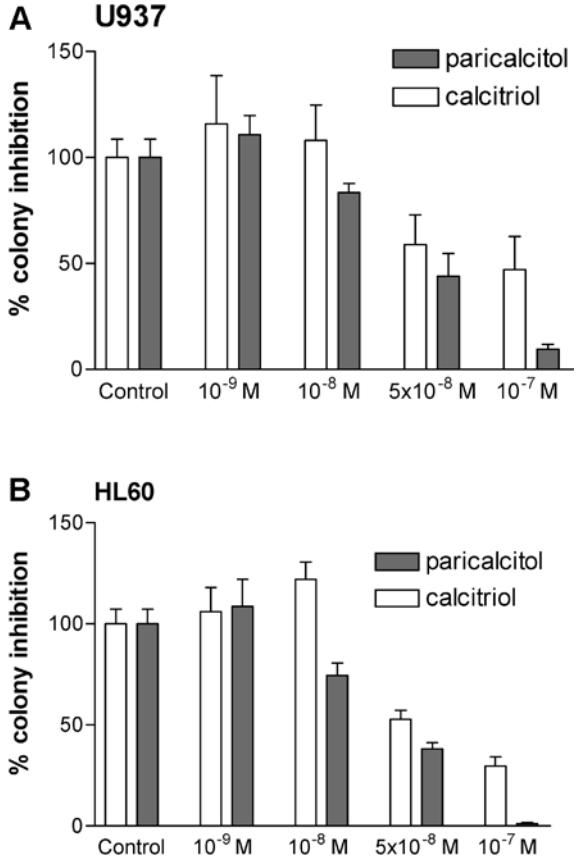

Fig. 5A, B Colony inhibition of leukemia cell lines by paricalcitol and calcitriol. Leukemic cells (U937 or HL-60) were cultured in triplicate in a methylcellulose semisolid medium and colonies of ≥40 cells were counted after 10 days (**A** U937 cells, **B** HL-60 cells). In each case (**A** and **B**), calcitriol and paricalcitol were added at 1% by volume at the indicated concentrations. The results are expressed as percentage in relation to the colonies of the control

Effect of paricalcitol on colony formation of normal bone marrow mononuclear cells

The effect of paricalcitol on colony formation of mononuclear cells obtained from a normal bone marrow did not differ from the effect of calcitriol. A modest increase in total colony numbers was observed at 10^{-9} *M* due to an increase in CFU-GM colony formation. At 10^{-8} *M* there was no change in the total colony count. At 10^{-7} *M*, a 50% decrease in colony count was seen with a more pronounced decrease in erythroid than myeloid colony formation (Table 2).

Discussion

We examined the effects of 19-nor-1α,25-dihydroxyvitamin D_2 on maturation, colony formation, apoptosis

Table 2 Colony formation of mononuclear cells from normal human bone marrow after treatment with paricalcitol and calcitriol. Bone marrow mononuclear cells were plated in triplicate in methylcellulose semisolid medium containing ethanol as control, calcitriol or paricalcitol (10^{-9}, 10^{-8} and 10^{-7} *M*) at 1% volume, and cultured for 14 days. CFU-GM, BFU-E and CFU-GEMM were counted. The mean ± SE numbers of colonies per plate are shown

	Control	10^{-9} *M*		10^{-8} *M*		10^{-7} *M*	
		Paricalcitol	Calcitriol	Paricalcitol	Calcitriol	Paricalcitol	Calcitriol
BFU-E	26 ± 1	25 ± 10	33 ± 7	15 ± 3	20 ± 3	0.7 ± 0.7	0
CFU-GM	65 ± 11	88 ± 17	86 ± 15	77 ± 10	71 ± 6	36 ± 6	48 ± 2
CFU-GEMM	3 ± 0.3	3 ± 1.7	5 ± 2.4	0	2.6 ± 0.6	0	0
Total	91 ± 12	113 ± 23	119 ± 12	91 ± 13	91 ± 7	37 ± 7	48 ± 2

induction, and cell cycle alterations in HL-60 and U937 leukemia cell lines. We also examined the effect of paricalcitol on normal bone marrow mononuclear cell colony formation. Paricalcitol induced differentiation of HL-60 and U937 cells, as determined by surface expression of CD11b antigen, and induced a concentration-dependent increase in the expression of CD14 antigen in HL-60, but not in U937 cells. Treatment with paricalcitol induced the appearance of cytoplasmic granules that were reduced NBT in HL-60, but not in U937 cells.

The reasons for the differing responses in the development of granules and CD14 expression in the HL-60 and U937 cell lines are unclear. However, $1,25(OH)_2D_3$ increases the number of cells that can reduce NBT (James et al. 1997b) and induces the expression of CD14 in U937 cells (James et al. 1997b; Rots et al. 1999). In a $1,25(OH)_2D_3$-treated U937 cell line, which expressed the promyelocytic leukemia zinc finger protein (PLZF), the morphological changes of differentiation and the expression of CD14 were blocked without blocking the induction of CD11b (Ward et al. 2001). PLZF is involved in a rare form of acute promyelocytic leukemia with the translocation t(11;17). The genes of PLZF and the retinoic acid receptor α (RARα) join in this reciprocal translocation, creating the PLZF-RARα fusion protein whose expression is sufficient to block differentiation by all-*trans*-retinoic acid (ATRA) and by $1,25(OH)_2D_3$ in both HL-60 and U937 cells. In our experiments, the effect of paricalcitol on U937 cells (overexpression of CD11b and no change in CD14) showed an expression pattern similar to that in $1,25(OH)_2D_3$-treated PLZF-expressing U937 cells, suggesting that paricalcitol may exert specific effects on the VDR function in these cells.

The maturation of the HL-60 cells after treatment with paricalcitol is similar to the effect of other 19-nor-vitamin D_3 compounds, which induce differentiation and inhibit the clonal proliferation of leukemia cell lines (Asou et al. 1998). Paricalcitol inhibited colony formation of HL-60 and U937 cells with an estimated IC_{50} of 3×10^{-8} *M* and 4×10^{-8} *M*, respectively. This effect was more potent than that obtained for calcitriol under the same experimental conditions (5×10^{-8} *M* for HL-60 and 8×10^{-8} *M* for U937 cells), suggesting that paricalcitol may be more effective against human myeloid leukemia than calcitriol ($P = 0.003$ in U937 cells, $P < 0.001$ in HL-60 cells).

Similar to calcitriol, paricalcitol increased the proportion of cells in G_0/G_1 phase and decreased the proportion of cells in S phase in a concentration-dependent manner after a 72-h exposure. This effect was more pronounced in HL-60 cells. Paricalcitol did not induce apoptosis in U937 cells. However, a significant increase in apoptosis was detected in HL-60 cells at 10^{-8} *M* and 10^{-7} *M* that was both time- and dose-dependent. In contrast, treatment with calcitriol alone does not induce apoptosis, despite downregulating bcl-2 protein levels (Elstner et al. 1996). Many other vitamin D analogues, including the 20-*epi*-vitamin D_3 analogues, lack the ability to induce apoptosis as single agents (Elstner et al. 1996; James et al. 1997a). The ability of paricalcitol to induce apoptosis in HL-60 cells resembles the effect of 21-(3-methyl-3-hydroxyl-butyl)-19-nor D_3 (Gemini-19-nor) (Hisatake et al. 2001). It is possible that the deletion of C-19 (the common characteristic of both analogues) is responsible for the effect of apoptosis induction in HL-60 cells when the cells are treated with these drugs alone. These findings support the view that, in HL-60 cells, the pathways for differentiation and apoptosis differ from one another (Zhang et al. 1994; Nagy et al. 1995; Albanell et al. 1996; Wang et al. 1996; Studzinski et al. 1997).

In summary, we observed that paricalcitol exerts antiproliferative and differentiating effects on HL-60 and U937 cells. Further investigations are needed to confirm that a selective inhibition of leukemic blasts can be achieved in vivo without toxicity. Because paricalcitol is already approved for use in humans (in renal disease), clinical trials with this calcitriol analogue could be undertaken readily.

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